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ANTIBODY DEVELOPMENT AND IMMUNOLIGAND ASSAY TESTING OF MS2 BACTERIOPHAGE ON THE LIGHT ADDRESSABLE POTENTIOMETRIC SENSOR

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The objectives of the work were to determine the compatibility and response of the antiserum with the virus; define the parameters of retaining viability for immunoligand assay testing upon purification and labeling procedures of the antiserum; and lastly, investigate the lower limit of detection (LOD) and sensitivity of the viral simulant on the LAPS.

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FOREWORD

Science and Technology Corporation (STC) is pleased to submit this report entitled "Antibody Development and Immunoligand Assay Testing of MS2 Bacteriophage on the Light Addressable Potentiometric Sensor" by Ms. Deborah G. Menking and Mr. Clifton R. Johnson of the STC Edgewood, Maryland office, and Mr. Samuel L. Eure of the STC Hampton, Virginia office.

PREFACE

The work described in this report was authorized under Contract No. DAAA15-89-D-0007, Delivery Order No. 0008, and Contract No. N00014-91-C-2185. This work was started in August 1990 and completed in July 1992.

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LIST OF ACRONYMS

ABTS	2,2 azino-di-3 ethyl-benzthozalin sulfate
BCD	BioChemical Detector
BSA	Bovine serum albumin
CRDEC	Chemical Research, Development and Engineering Center
DNP	dinitrophenyl
DPG	Dugway Proving Ground
ELISA	Enzyme-linked immunosorbent assay
h-NHS	Hapten-N-hydroxysuccinimide ester
HPLC	High performance liquid chromatography
HRPO	Horseradish peroxidase
IgG	Immunoglobulin G
ILA	Immunoligand assay labeling kit
LAPS	Light-addressable potentiometric sensor
LOD	Limit of detection
MAPS II	Monoclonal Antibody Purification System
MCR	Molar coupling ratio
MDC	Molecular Devices Corporation
MIR	Molar incorporation ratio
MS2	Viral simulant in BCD program
NHS	N-hydroxysuccinimide
ONPG	o-nitrophenyl β-D-galactopyranoside
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
pH	phosphate
STC	Science and Technology Corporation
UV	Ultraviolet

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ANTIBODY DEVELOPMENT AND IMMUNOLIGAND ASSAY TESTING
OF MS2 BACTERIOPHAGE ON THE LIGHT ADDRESSABLE POTENTIOMETRIC SENSOR

1. INTRODUCTION

The U. S. Army Chemical Research, Development and Engineering Center (CRDEC) tasked Science and Technology Corporation (STC) to thoroughly screen and evaluate the efficacy of selected candidate antibodies against the bacteriophage MS2 for possible assay development of a viral simulant on the BioChemical Detector (BCD). The viral simulant in the BCD program, MS2, is a bacteriophage from *Escherichia coli* . In the electron microscope MS2 appears to be a small polyhedral object, about 26 millimicrons, having a particle weight of 3.6 x 10^6. The virus was originally isolated by Dr. Alvin J. Clark and has a very unusual and compact ribonucleic acid (RNA) structure. Considering the phosphorus content, the bacteriophage is estimated to be 32% RNA by weight (Strauss and Sinsheimer, 1963).

Specifically, two monoclonal cell lines, 5A4-1 and 3A2-12, and polyclonal rabbit serum were investigated as possible considerations for assay development on the light-addressable potentiometric sensor (LAPS). The objectives of this work were to determine the compatibility and response of the antiserum with the virus; define the parameters of retaining viability for immunoligand assay testing upon purification and labeling procedures of the antisera; and lastly, investigate the lower limit of detection (LOD) and sensitivity of the viral simulant on the LAPS.

Quantitative detection of MS2 was performed using immunoligand assays (ILA) with the LAPS which is manufactured by Molecular Devices Corporation (MDC) of Menlo Park, California. The LAPS is an immunoassay instrument which performs one-step assays that can be completed in a short period of time (Briggs, 1987; Hafeman and McConnell, 1988; and Parce, et al., 1989). The LAPS technology uses a specially coated biotinylated nitrocellulose membrane (0.45- and 5-μm pore size) onto which fluoresceinated immunocomplexes are immobilized followed by reaction with an anti-fluorescein urease-conjugated antibody. Analyte quantitation is accomplished by placing the urease-labeled immunocomplex into a reader containing a 100-mM solution of urea. Hydrolysis of the urea produces a potentiometric shift proportional to the amount of urease present in solution and, hence, sensitive and reliable detection of the target protein (Molecular Devices Corp., 1990).

Included in the text material is a compilation of the procedures, data, and evaluations for the task effort. Technical data includes both positive and negative results along with pertinent information and conclusions concerning the efficacy of the selected antibodies.

2. MATERIALS AND METHODS

2.1 ACQUISITION OF ANTISERA AND MS2 BACTERIOPHAGE

Anti-MS2 ascites (5A4-1 and 3A2-12) and rabbit polyclonal serum were obtained from the Baker Laboratory at Dugway Proving Ground (DPG), Utah. The MS2 viral antigen was also obtained from DPG with an assay titer of 6.8×10^{10} organisms per ml. Anti-fluorescein urease enzyme reagent was purchased from MDC in lyophilized form and reconstituted with 30 ml of assay reagent buffer and stored at 4°C.

2.2 BUFFER PREPARATIONS AND BINDERS USED

Preparations of 10-mM wash/substrate buffer (pH 6.5) and 10-mM assay reagent buffer (pH 7.0) were prepared from commercially obtained buffer concentrates from MDC. Both concentrates were diluted 1:10 with deionized water and sterile-filtered through a 0.22-μm filter and stored at 4°C for several weeks. A 100-mM reader substrate solution was made fresh daily by adding 0.6 gm of urea to 100 ml of wash/substrate buffer. Streptavidin (#21122) was purchased from Pierce (Rockford, Illinois) at 1 mg per ml. Nitrocellulose-coated membrane sticks with 5-μm pores were purchased from MDC and used for the duration of the assay testing.

2.3 PREPARATION OF ANTI-MS2 ASCITES AND SERUM

Before incorporating the anti-MS2 antibody into an assay, it was necessary to screen and characterize the antibody for protein quantitation, viability, and purity. It was paramount to know the condition and response of the antibody through each step of sensor preparation.

2.3.1 Protein Quantitation

Protein concentration was determined by two methods: ultraviolet (UV) detection on the DU 7000 spectrophotometer (Beckman), and BIO-RAD'S Bradford Assay. Absorbance of UV irradiation by protein was the quickest method for quantitating antisera solutions. Readings were performed at 280 nm for murine immunoglobulin G (IgG) and calculated by the following equation:

$$mg/ml = \frac{A_{280} \times dilution\ factor}{1.37}$$

The absorbance at 280 nm was multiplied by the dilution factor and divided by 1.37, which is the extinction coefficient for murine IgG.

BIO-RAD has designed a commercial protein concentration determination kit based on the Bradford test. The Bradford dye concentrate was Coomassie brilliant blue G-250 in 95% ethanol. Protein samples were made using Bovine serum albumin (BSA) for a standard curve. Anti-MS2 test samples were prepared and diluted-dye binding solution was added to each sample. After an incubation time of 15 min at room temperature, the absorbance was read at 595 nm.

2.3.2 Protein Viability

Enzyme-linked immunosorbent assays (ELISA) were used to quantitate the viability and interaction of the anti-MS2 antibody with its corresponding antigen. Three types of ELISAs were used for testing antibody/antigen interactions: antibody capture assays, antigen capture assays, and two-antibody sandwich assays.

For the antibody capture assay, the MS2 was attached to the solid support (polystyrene microtiter plate) and the labeled anti-MS2 was allowed to bind. After incubation and washing, the assay was quantitated by a measurement of the amount of antibody retained on the solid support. The antigen capture assay was identical to the above; however, only the antigen and antibody placement were reversed. In the two-antibody sandwich assay, the capture antibody was bound to the solid support, and the MS2 antigen was allowed to bind to the first antibody. The assay was quantitated by a measurement of the amount of the labeled second antibody that bound to the MS2 antigen.

2.3.3 Protein Purity

The anti-MS2 antibody was supplied as ascites fluid and serum preparations. Before these reagents could be used in detection assays, they had to be purified of contaminants and extraneous substances which would interfere with the labeling efficiency of the antibodies.

Both anti-MS2 monoclonal cell lines and polyclonal serum were purified by affinity chromatography on a 10-cm column with 5 ml of Protein A agarose gel beads from BIO-RAD's Affi-Gel Monoclonal Antibody Purification System (MAPS II). The column was equilibrated with a proprietary binding buffer solution, pH 9.0, supplied in the kit. One milliliter of antibody was combined 1:1 with a binding buffer and placed on the column. The flow rate was set at 0.5 ml per min, and the IgG portions were eluted with an elution buffer at pH 3.0. The IgG fractions were immediately neutralized with a 1-M Tris buffer, pH 10.6. Typical protein recoveries were 7 to 8 mg of IgG[1] from 1-ml applications of both ascites and polyclonal protein. The IgG fractions were pooled and dialyzed overnight in 0.1-M phosphate buffered saline (PBS), pH 7.0. Because of the resultant high percentage of protein recovery and high level of purity, this separation technique was chosen specifically for separation of IgG from anti-MS2 ascites and serum.

To assess the purity of the anti-MS2 samples, Pharmacia's PhastSystem Native polyacrylamide gel electrophoresis (PAGE) was chosen. Anti-MS2 samples were transferred to a membrane on gradient gels 8-25 (50 to 600 kD) and 10-15 (90 to 600 kD) and run with corresponding low and high molecular weight standards. The protein purity and molecular weight were determined by this technique. The gels demonstrated single IgG bands of anti-MS2 at approximately 160,000 Daltons (D).

2.4 PREPARATION OF ANTI-MS2 CONJUGATES

After the anti-MS2 antibody was screened and purified, it was coupled to haptens that completed the reaction complexes that were necessary in the assay format for the biosensor. Anti-MS2 biotin and fluorescein conjugates were made by reaction of the antibodies with N-hydroxysuccinimide (NHS) esters of dinitrophenyl-(DNP) biotin and carboxyfluorescein using the Immunoligand Assay Labeling Kit (ILA) purchased from MDC. The protocol MDC supplies for the ILA was followed to determine which protein to label, the amounts of Hapten-N-hydroxysuccinimide ester (h-NHS) to use, the amounts of protein to label, and the molar coupling ratios (MCR) to use. Recommended target molar incorporation ratios (MIRs) were 4 to 5 moles of biotin and 3 to 6 moles of fluorescein per mole of protein. After the labeling procedure, the anti-MS2 conjugates were purified on Pharmacia's PD-10 Sephadex G-50 column. After labeling, the anti-MS2 conjugates were diluted 1:1 with assay reagent buffer to prevent loss of viability because of the presence of proteases. The immunoreactants were then tested for activity using ELISA.

Labeling responses and assay results (using 0.45-μm pore-size assay sticks) of anti-MS2 monoclonal cell line 5A4-1 and anti-MS2 polyclonal have previously been reported [6]; however, modifications in assay configuration for both antisera mentioned above as well as testing of the monoclonal cell line 3A2-12 will be reported in this paper.

2.5 ELISA TESTING

ELISA tests were conducted to determine the specificity and activity of the anti-MS2 monoclonal and polyclonal antibodies to the MS2 antigen. The anti-MS2 conjugates were labeled and stablized; however, it was necessary to know the antibody titer. ELISA tests were conducted to determine the specificity and activity of the anti-MS2 monoclonal and polyclonal antibodies to the MS2 antigen.

2.5.1 ELISA Testing of Unlabeled Anti-MS2 Antibodies

The ELISA protocol for the tests was a simple sandwich assay with MS2 coated in glycine at 1:1000 on a 96-well polystyrene Dynatech microtiter plate. After an overnight incubation at 4°C, the plates were washed with 0.1-M phosphate buffered saline (PBS) with Tween 20. Because the IgG concentration of the ascites and serum were unknown, they were diluted 1:100, and the purified antibody samples were added at 5 μg per ml and titered across the wells at 1:2. After a 1-hr incubation at 37°C and washing, anti-mouse (monoclonal 5A4-1 and 3A2-12) and anti-rabbit (polyclonal) horseradish peroxidase (HRPO) was added to the plate at 1:1000. One final incubation and washing was performed and the substrate 2,2 azino-di-3-ethyl-benzthozalin sulfate (ABTS) was added. The plate was read at 405 nm after a 30-min incubation.

2.5.2 ELISA Testing of Anti-MS2 Antibodies Conjugated to Biotin

The ELISA designed to test for the presence of biotin was an antibody capture test using anti-MS2 monoclonal 5A4-1 and 3A2-12, and polyclonal antibody samples conjugated to biotin as described in Section 2.4. A 96-well Dynatech polystyrene microtiter plate was coated with antibody-biotin samples in a glycine coating buffer at 5 μg per ml, titered across at 1:2, and allowed to incubate overnight at 4°C. After washing the plate with PBS with Tween 20, streptavidin beta-galactosidase was added at 0.2 μg per ml and allowed to incubate for 1 hr at 37°C. The plate was again washed, and the substrate o-nitrophenyl beta-D-galactopyranoside (ONPG) was added. The plate was read at 405 nm after a 30-min incubation.

2.5.3 ELISA Testing of Anti-MS2 Antibodies Conjugated to Fluorescein

The ELISA test conducted to determine the presence of fluorescein was an antibody capture test using anti-MS2 monoclonal 5A4-1 and 3A2-12, and polyclonal antibody samples conjugated to fluorescein as described in Section 2.4. A 96-well Dynatech polystyrene microtiter plate was coated with antibody-fluorescein samples in a glycine-coating buffer at 5 μg per ml, titered across at 1:2, and allowed to incubate overnight at 4°C. After washing the plate with PBS with Tween 20, an MDC anti-fluorescein urease antibody was added at 1:10 (150 ng per well) and allowed to incubate for 1 hr at 37°C. The plate was again washed, and the substrate bromocresol purple was added. The absorption of urease is at 690 nm; however, because of the lack of a filter for 690-nm readings, a microtiter plate containing bromocresol purple was read at 450 nm to serve as a blank. After a 30-min incubation, the test plate was read at 450 nm and the optical density was determined by subtracting the numerical value of the test plate from the blank plate value to achieve the optical density for the presence of fluorescein.

2.5.4 ELISA Test of Anti-MS2 Antibodies to Determine Availability of Binding Asites

Once ELISA testing confirmed the successful labeling of the antibodies, it was necessary to further test if the binding sites on the antibody were free or blocked. ELISA tests were designed using an antigen capture assay for biotinylated and fluoresceinated antibodies. Two 96-well polystyrene Dynatech microtiter plates were coated with the MS2 virus at 10^8 to 10^1 organisms in a glycine coating buffer and allowed to incubate overnight at 4°C. Both plates were washed with PBS with Tween 20. Anti-MS2 biotinylated antibodies (plate 1) and fluoresceinated antibodies (plate 2) were added 1:100 in diluent to the bound MS2 on the plate and incubated for 1 hour at 37°C. After washing the plates, streptavidin beta-galactosidase was added at 0.2 μg per ml to plate 1 (containing the bound biotinylated antibody), and the anti-fluorescein urease antibody was added at 1:10 to plate 2 (containing the bound fluoresceinated antibody) and allowed to incubate for 1 hr at 37°C. After washing the plates, substrate ONPG was added to plate 1, and substrate bromocresol purple was added to plate 2 and allowed to incubate for 30 min. Plate 1 was read at 405 nm, while plate 2 was read at 450 nm.

2.6 ANTIGEN PREPARATIONS FOR LAPS ASSAY

The MS2 viral antigen was diluted from stock (6.8 x 10^{10} organisms per ml) with an assay reagent buffer to a starting concentration of 10^9 organisms per ml. Serial dilutions were made to produce a final concentration range of 10^8 to 10^1 organisms per well.

2.7 DETECTION ASSAY PROTOCOL FOR LAPS ASSAY

A blank stick was run before each day's work. The vacuum settings were set at 3 (low) and 40 (high). Assays were run on 5-μm sticks with one well reserved for background readings (zero antigen present). The remaining 7 wells contained aliquots of sample antigen from 10^8 to 10^2 organisms per well.

2.8 ANTIBODY COCKTAIL MIXES FOR LAPS ASSAY

The antibody cocktail mixes consisted of an antibody reagent mix made of anti-MS2 biotin (5 μl of a 0.2 mg per ml concentration), anti-MS2 fluorescein (5 μl of a 0.2 mg per ml concentration), and streptavidin at 5 μg per ml (5 μl of a 1 mg per ml concentration) combined with 985 μl of assay buffer to total 1 ml. The assay procedure required that an aliquot of 100 μl each of MS2 sample dilution placed into one of seven borosilicate tubes. To each was added 100 μl of antibody cocktail mix t final antibody concentration of 50 ng per well (tests were also run at 10, 100, and 200 ng concentrations of antibody). The complex was incubated for 5 min. Meanwhile, 500 μl of wash per substrate solution was added to each filter well and vacuumed on high for approximately 1 min. After the incubation period, 100 μl from each test tube was immediately delivered to the assembled filter unit and filtered on low for approximately 1 min. To each of the eight wells on the filter unit, 100 μl of enzyme reagent was added and filtered on low. The wash per substrate buffer (500 μl) was added to each well and washed on high for 1 min. The filtered membrane stick was then removed and placed in the reader for data acquisition.

3. RESULTS

3.1 ELISA TESTING OF THE ANTI-MS2 ANTIBODY

Figures 1 and 2 show the positive response curves of the three antisera to the MS2 virus. Once the ELISA tests demonstrated the affinity of the monoclonal and polyclonal antibodies for the antigen, it was then necessary to test the viability and response of the antibodies conjugated to biotin and fluorescein to the MS2.

Figure 3 shows the response of the biotinylated 5A4-1 and 3A2-12 monoclonal antibodies to determine the presence of biotin and the conjugate interaction with MS2 antigen.

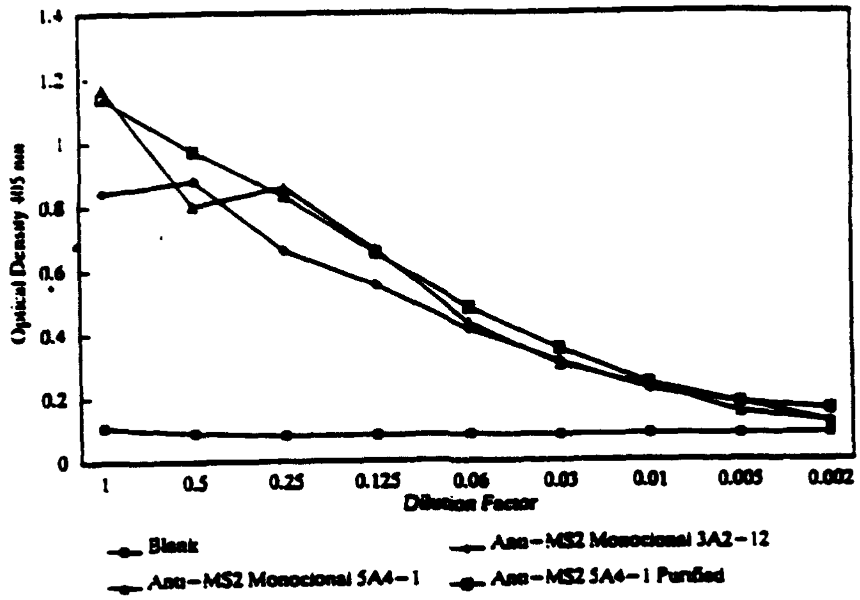

Figure 1. ELISA response curve of 5A4-1 and 3A2-12 monoclonal antibody to MS2 antigen.

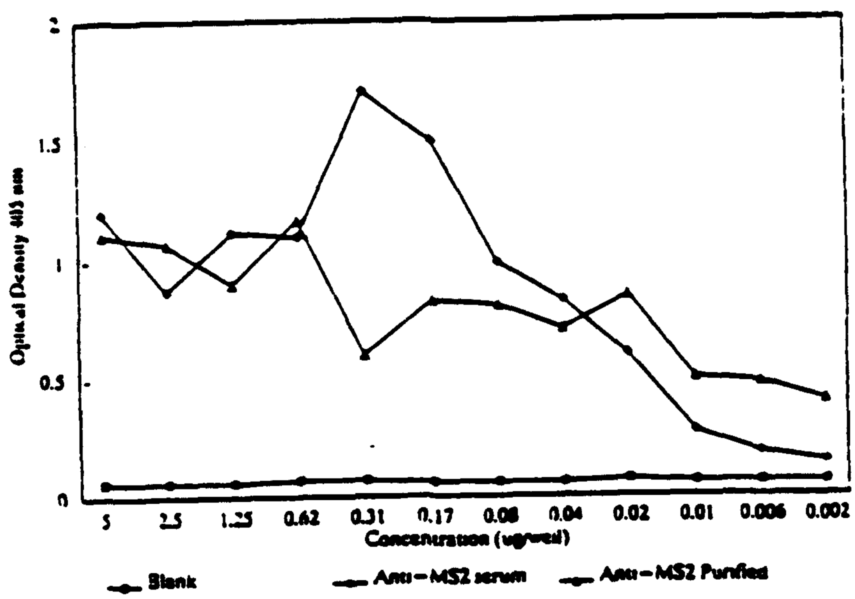

Figure 2. ELISA response curve of polyclonal rabbit serum to MS2 antigen.

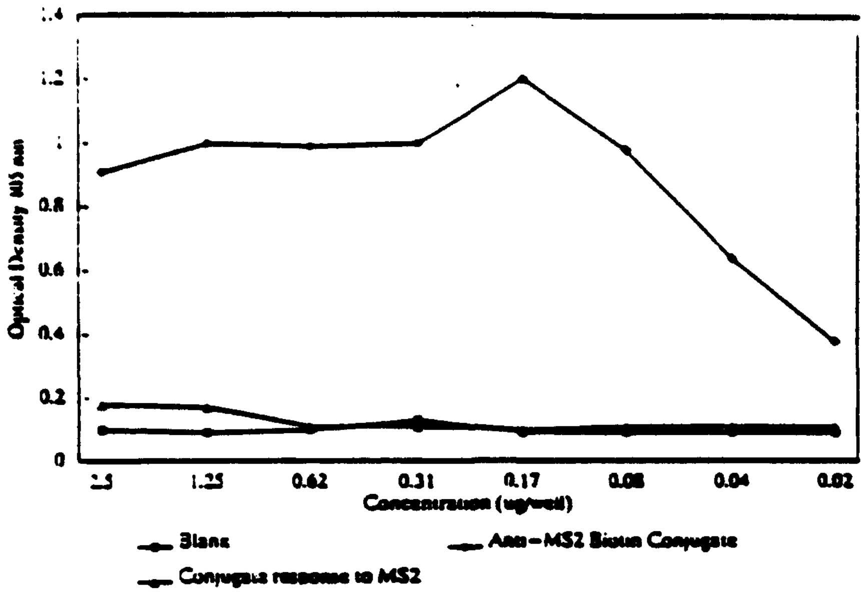

Figure 3. ELISA response of biotinylated 5A4-1 and 3A2-12 monoclonal antibodies to determine the presence of biotin and its interaction with MS2 antigen.

Figure 4 shows the response of the biotinylated rabbit polyclonal antibody and its interaction with the MS2 antigen. In both cases, the monoclonal and polyclonal antibodies exhibited positive results with the presence of available biotin. However, when assayed in the presence of the MS2, only the polyclonal antibody responded favorably to the antigen, indicating that the active binding sites on the biotinylated monoclonal antibodies were limited and had been blocked or sterically hindered by the labeling procedure.

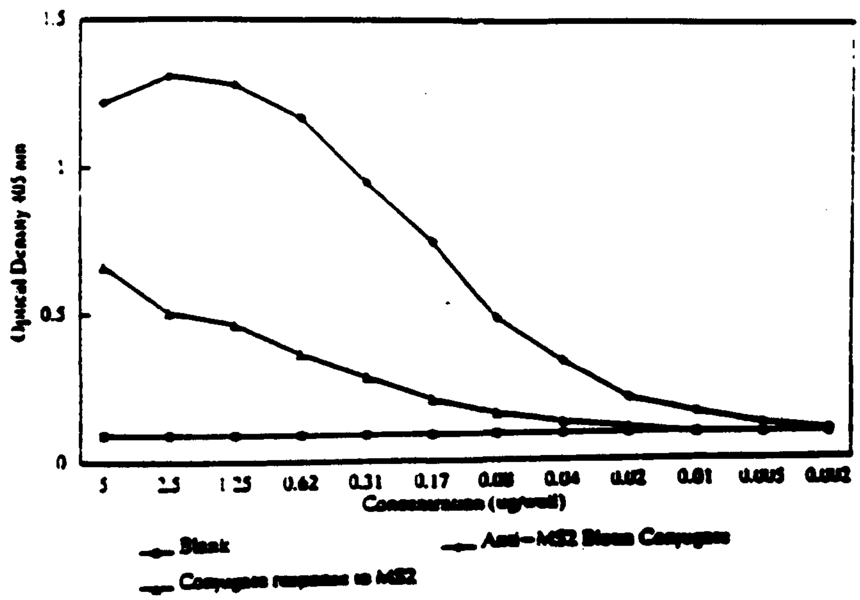

Figure 4. ELISA response of biotinylated polyclonal rabbit to determine the presence of biotin and its interaction with the MS2 antigen.

Figure 5 demonstrates the response of the fluoresceinated 5A4-1 and 3A2-12 monoclonal antibodies and polyclonal to determine the presence of fluorescein and the conjugate interaction with MS2 antigen. Again, both monoclonal and polyclonal antibodies responded well to labeling with fluorescein, but, as in the case with the biotin labeling, only the polyclonal antibody recognized the antigen when tested.

Repeated efforts were made to label the monoclonal antibodies with biotin and fluorescein at lower MIRs to enhance the possibility of available binding sites for the MS2. Extensive ELISA sandwich assays were conducted combining only the monoclonal antibodies in every possible configuration for an assay, but the results were negative.

In dealing with the problems of limited binding sites and steric hindrance of the labeled antibody, an ELISA was designed that sandwiched the MS2 antigen between a biotinylated polyclonal antibody used as the capture antibody while a fluoresceinated monoclonal antibody (5A4-1 or 3A2-12) was used as the detector antibody. Figure 6 shows the result of the sandwiched antibodies when assayed at 1:100

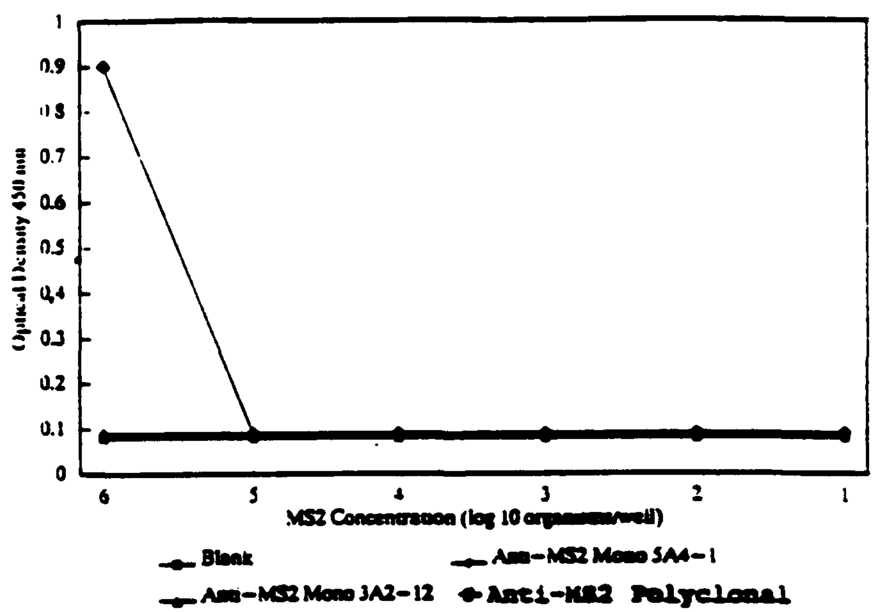

Figure 5. ELISA response of fluoresceinated 5A4-1 and 3A2-12 monoclonal and polyclonal antibodies to determine the presence of fluorescein and its interaction with the MS2 antigen.

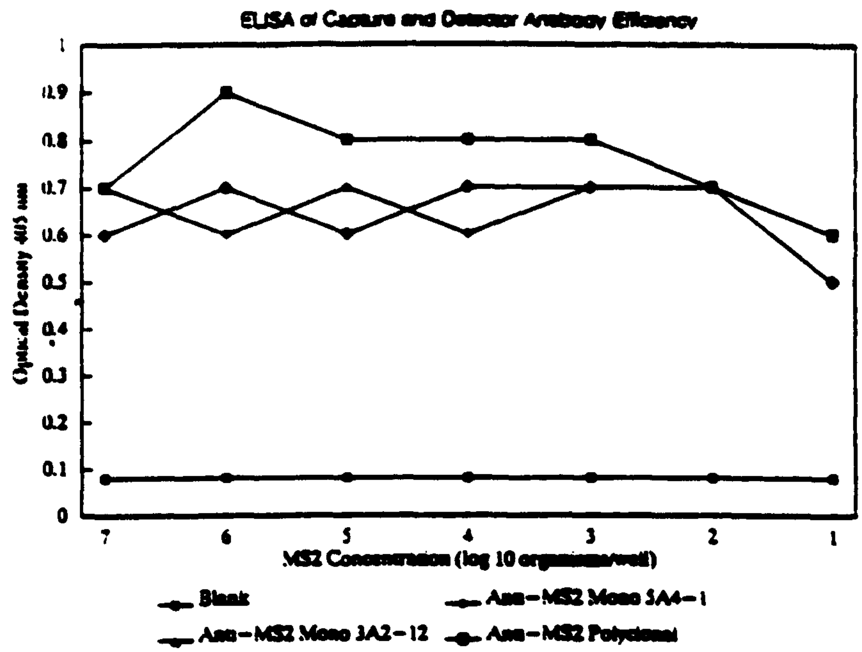

Figure 6. ELISA test to determine the feasibility of sandwiching the biotinylated polyclonal capture antibody with the fluoresceinated monoclonal 5A4-1 detector antibody.

applications with the MS2 antigen. The results were positive and demonstrated the success of immobilizing the rather versatile biotinylated polyclonal antibody to the solid matrix while allowing sufficient binding to the virus and subsequent binding of the MS2 to the fluoresceinated monoclonal antibody.

With this assay information at hand, it was then necessary to design an ELISA that would test the conjugates using a LAPS assay format. To accomplish this, it was necessary to repeat the above assay using the anti-fluorescein urease antibody (used in LAPS assays) to tag the fluoresceinated monoclonal antibodies. Figure 7 shows the response of the conjugates in the LAPS assay format and demonstrates the recurring problem of steric hindrance and competition for binding sites of the monoclonal conjugates when in the presence of the MS2 virus and a large molecule such as the anti-fluorescein urease antibody. Only the polyclonal biotin/MS2/polyclonal fluorescein conjugate sandwich combination showed positive response for the assays. Sensitivity at 10^7 organisms was described for all polyclonal assay testing.

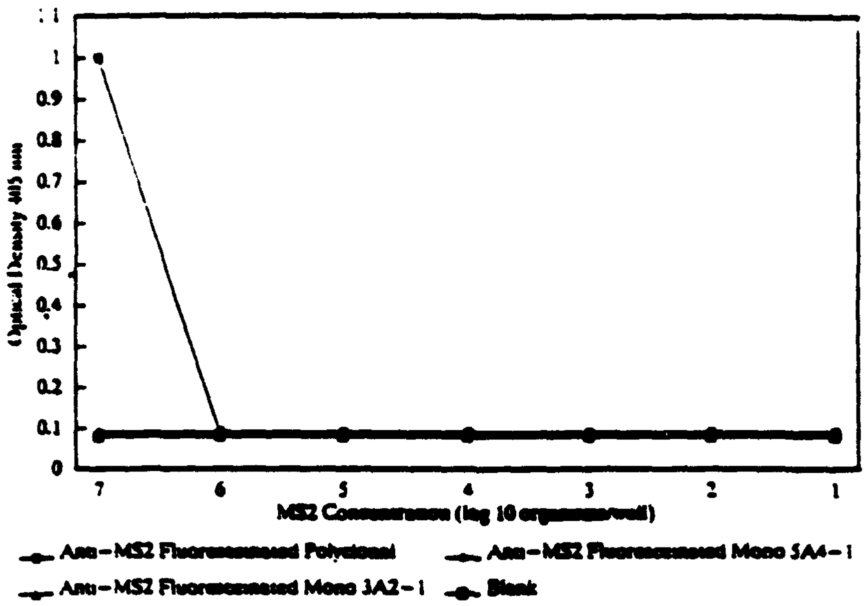

Figure 7. ELISA sandwich assay using LAPS assay format with the anti-fluorescein urease antibody.

3.2 LAPS TESTING OF THE ANTI-MS2 CONJUGATES

Figure 8 shows the quantitative detection for MS2 using polyclonal conjugates on the LAPS. The results correspond to the ELISA data that demonstrates a lower limit of detection of 10^7 organisms.

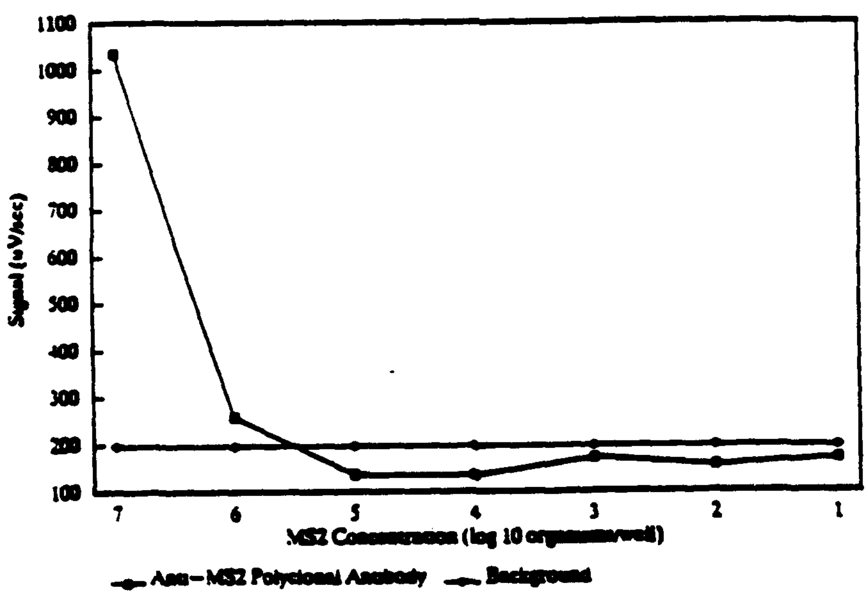

Figure 8. Quantitative detection on LAPS of MS2 assay with a 5-minute incubation period.

Additional LAPs assays were conducted using a combination scheme of the biotinylated polyclonal antibody (50 and 100 ng concentrations tested) sandwiched with fluoresceinated monoclonals 5A4-1 and 3A2-12 (50 and 100 ng concentration used). A positive response was demonstrated with the biotinylated polyclonal and fluoresceinated monoclonal 5A4-1 combination at 30-min and 1-hr incubations. Background values were higher than normal, but signal values at 10^7 organisms were significant above background and suggested that further optimization of the 5A4-1 monoclonal assay might yield favorable results, but only with lengthened incubation periods. Assays using 3A2-12 conjugate combinations continued to give negative results.

4. DISCUSSION

The bacteriophage MS2 is a small virus, originally isolated by Dr. Alvin J. Clark, which has an unusual RNA structure that is very compact. The virus has a particle weight of 3.6×10^6, and from the phosphorus content is 32% RNA by weight.

Purification of anti-MS2 ascites and serum was performed successfully by Protein A affinity chromatography with excellent IgG recoveries of 7 to 8 mg per ml of antisera. Native PAGE and diagnostic testing on Gilson's high-performance liquid chromatography (HPLC) equipment revealed pure IgG bands and single peaks.

Efforts to label the purified monoclonal 5A4-1, 3A2-12, and rabbit polyclonal antibody at target molar incorporations for ILA assays proved very successful when ELISA tested for the presence of the haptens. Antibody conjugates were stored in the assay reagent buffer after labeling to eliminate (the tendency of) the formation of particulate matter after 5 days.

Although the monoclonal and polyclonal antibody conjugates responded well in ELISA testing designed to determine the presence of the haptens, only the polyclonal biotin (MIR 4) and fluorescein (MIR 3) conjugates exhibited reproducible assay responses at 10^7 MS2 organisms when ELISA tested and LAPS tested (5-minute incubation period for LAPS assays). Positive LAPS response was demonstrated with a sandwich combination of biotinylated polyclonal antibody (MIR 4) used as the capture antibody and fluoresceinated monoclonal 5A4-1 conjugate (MIR 6) used as the detector antibody for 30-min and 1-hr incubations. Numerous attempts were made to design feasible assay combinations of monoclonal

5A4-1 and 3A2-12 conjugates at various MIRs for LAPS testing, but negative results were exhibited for each test.

In this report an effort has been made to characterize and screen three anti-MS2 antibodies for assay development on the BCD. Based on the results from ELISA and LAPs assays of the anti-MS2 antibodies, the rabbit polyclonal antisera demonstrates reproducible assays with a sensitivity of 10^7 organisms for 5-min ILA assays. Increasing the incubation time to 30 min and 1 hr did not give a lower sensitivity. The monoclonal cell lines 5A4-1 and 3A2-12 do not respond to 5-min and 1-hr incubation periods for ILA assays on the LAPS because of steric hindrance after conjugation to biotin and fluorescein.

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